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In Vitro Culture of Turkish *Origanum sipyleum* L.

Başar Sevindik[1], Tolga İzgü[2], Özhan Şimşek[1], Mehmet Tütüncü[3], Pembe Çürük[1], Özer Yılmaz[4], Gönül Kaynak[4], Yıldız Aka Kaçar[1], Jaime A. Teixeira da Silva[5], Yeşim Yalçın Mendi[1, *]

[1]Department of Horticulture, Faculty of Agriculture, University of Çukurova Balcalı, Adana, Turkey

[2]Department of Horticulture, Faculty of Agriculture, University of Ege Bornova, İzmir, Turkey

[3]Department of Horticulture, Faculty of Agriculture, University of Ondokuz Mayıs Atakum, Samsun, Turkey

[4]Department of Biology, Faculty of Arts and Sciences, University of Uludağ, Nilüfer, Bursa, Turkey

[5]Miki-cho Post Office, Ikenobe, Kagawa-ken, Japan

Email address:

basarsevindik@gmail.com (B. Sevindik), tolgaizgu01@gmail.com (T. İzgü), ozhan12@gmail.com (Ö. Şimşek), mtutuncu.tr01@gmail.com (M. Tütüncü), pcuruk@cu.edu.tr (P. Çürük), ozery@uludag.edu.tr (Ö. Yılmaz), gkaynak@uludag.edu.tr (G. Kaynak), yildizakakacar@gmail.com (Y. A. Kacar), jaimetex@yahoo.com (J. A. Teixeira da Silva), yesimcan@cu.edu.tr (Y. Y. Mendi)

[*]Corresponding author

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Abstract: *Origanum sipyleum* L. is a species that is endemic to Turkey with known medicinal and aromatic properties and with a long historical use in Turkish folk medicine. Micropropagation serves as one possible method to clone and protect *O. sipyleum.* In this study, nodes, which served as the explants, were cultured on Murashige and Skoog (MS) basal medium supplemented with different concentrations of BA (0.1, 0.5, 1.0 mgL^{-1}) and GA_3 (0.1, 0.2 mgL^{-1}). Shoots were transferred to MS medium containing different concentrations of IBA (0, 0.5, 1, 1.5, 2, 2.5, 3 mgL^{-1}) for root induction. Shoot regeneration, rooting, survival and acclimatization were possible. As many as 85% of nodes formed an average of 6 shoots/node on MS medium supplemented with 0.5 mgL^{-1} BA and 0.2 mgL^{-1} GA_3. Rooting was most successful in 62.5% of shoots on MS medium with 1.5 or 2.5 mgL^{-1} IBA. Plantlets derived from all IBA combinations were transferred to autoclaved peat and perlite (1:1, v/v) and acclimatized in a greenhouse, with 79.1% survival. This protocol represents the first comprehensive *in vitro* regeneration protocol for endemic (Turkish) *O. sipyleum*.

Keywords: BA, GA_3, IBA, MS Medium, Tissue Culture

1. Introduction

The *Lamiaceae* contains nearly 50 genera, 546 species and 730 taxa [1]. Of these, 23 species and 32 taxa occur in Turkey [2], including many species with culinary, medicinal and health purposes, including *Origanum* spp. [3]. *Origanum* species are mainly herbaceous perennials or subshrubs that are native to the Mediterranean and Eurasia, and grow in mountainous areas in rocky, calcareous soil [1]. The spice oregano is derived from *Origanum* species [4]. Although the dominant secondary metabolites of *Origanum* spp. are carvacrol and thymol, each species contains different amounts of these chemical compounds [5]. The essential oil and stimulants extracted from *Origanum* species are also important [6]. The essential oil from *O. sipyleum* L. contains 48 compounds, the most common being γ-terpinene, *p*-cymene, thymol methyl ether, carvacrol methyl ether, thymol and carvacrol [7]. These compounds are effective in the cure of hypoglycaemia [8], especially from carvacrol-rich species (*O. heracleoticum* L., *O. maru* L., and *O. smyrnaeum* L.), which are used as spices in Turkey. *O. sipyleum* forms an 80 cm tall perennial semi-shrub with pink flowers, prefers warm climates and grows well in arid rocky calcareous soils rich in nutrients, similar to other *Origanum* species [9]. *O. sipyleum* is endemic to Western Anatolia, flowers from April to October, has an altitudinal distribution between 100 and 1500

m in western and southern Anatolia and a 3-4 year life span when grown under favourable climatic conditions [1].

Tissue culture and micropropagation has been achieved in some *Origanum* species (Table 1). One study exists for *O. sipyleum* in which Oluk and Çakir (2009) used nodes from seedlings as explants in micropropagation. However, these authors collected *O. sipyleum* seeds from Spil Mountain in Manisa, in Western Turkey [10].

In the present study, native *O. sipyleum* plants were sampled from Uludağ Mountain in Bursa, in the north-western part of Turkey. Nodes were also used as the explant source with the objective of establishing a protocol to micropropagate plantlets in the presence of 6-benzyladenine (BA) and gibberellic acid (GA_3).

Table 1. *Micropropagation studies in different Origanum species.*

References	*Origanum* species	Explant	Basal medium	Best PGR concentrations for shoot induction	Best conditions for rooting
[22]	*O. vulgare* L.	Shoot and individual seedling	MS	1 mgL^{-1} BA	PGR-free MS
[20]	*O. bastetanum* L.	Nodal segment with two axillary buds	Undefined	1 mgL^{-1} BA	*In vivo* rooting
[14]	*O. vulgare* subsp. *hirtum*	Shoot with axillary bud	B5	0.01 mgL^{-1} NAA	B5 medium + 0.01 mgL^{-1} NAA
[21]	*O. syriacum* L.	Internode segment	MS	0.4 mgL^{-1} Kin + 0.8 or 1.2 mgL^{-1} TDZ	MS + 0.8 mgL^{-1} IAA
[19]	*O. vulgare* L.	Shoot tip	MS	0.76 mgL^{-1} BA + 0.01 mgL^{-1} NAA	Spontaneous rooting
[23]	*O. vulgare* L. *O. syriacum* L.	Internode segment	MS	1.5 mgL^{-1} TDZ with or without 0.5 mgL^{-1} 2, 4-D (callus formation)	-
[12]	*O. minutiflorum*	Single nodal	MS	2 mgL^{-1} BA + 0.1 mgL^{-1} NAA	MS + 3.0 mgL^{-1} IBA
[16]	*O. vulgare* L. ssp. hirtum	Axillary bud	MS	1 mgL^{-1} BA	Spontaneous rooting
[10]	*O. sipyleum* L.	Shoot tip	Modified MS	1 mgL^{-1} BA	MS with 0.5 mgL^{-1} IBA
[4]	*O. acutidens* (Hand.-Mazz.) Ietswaart	Stem node	MS	1.8 mgL^{-1} BA + 0.2 mgL^{-1} NAA	MS + 0.2 mgL^{-1} NAA

BA, 6-benzyladenine; IBA, indole-3-butyric acid; Kin, kinetin; MS, Murashige and Skoog [11] medium; NAA, 1-naphthalene acetic acid; TDZ, thidiazuron

2. Material and Methods

2.1. Plant Material

A total of 60 plants were collected from Uludağ mountain (NW Turkey) (40°05'27.55" N (long.), 29°10'26.26" E (lat.), 1600 m above sea level) during August, 2012. Plants were cultivated individually in pots with peat, sand and perlite (1:1:1, v/v/v) in the ornamental plant greenhouses (30°C; 2-11 MJ m^{-2} day^{-1}) at Çukurova University and irrigated three times a week with tap water each morning.

2.2. Surface Sterilization and Culture Conditions

Shoots including apical buds from one-year-old plants and 2 cm in length were washed under tap water for 30 min. Explants were dipped for 20 min in 0.1% $HgCl_2$ (Merck KGaA, Darmstad, Germany) in a fume hood. Nodes were rinsed five times with distilled water, dipped in 70% ethanol for 1-2 min, soaked in 20% NaOCl (4.6% active chlorine) (Domestos, Unilever, Turkey) for 20 min, then rinsed with sterilized distilled water 4-5 times in a sterile cabinet.

Explants were cultured on Murashige and Skoog (MS) [11] (Duchefa RV, Haarlem, the Netherlands) basal medium containing 3% sucrose, 7.5 gL^{-1} agar (Duchefa, P1001) and different concentrations of BA (0, 0.5, 1.0, 1.5, 2.0, and 2.5 mgL^{-1}) and GA_3 (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mgL^{-1}). The pH of media was adjusted to 5.6-5.7 by 1 N KOH and 1 N HCl. Explants were placed into test tubes (one/tube) and incubated at 25°C, 40 µmol m^{-2} s^{-1} (F36W/54-765 daylight 2350Lm, Philips Lighting Holding B.V.) and a 16-h photoperiod. Explants were subcultured into glass tubes (15 cm, Sigma-Aldrich, St. Louis, MI, USA) containing 15 mL of same medium every 4 weeks for a total of two subcultures. Shoots derived from nodes were then transferred to rooting medium, which contained different concentrations of indole-3-butyric acid (IBA) (I0902, Duchefa) (0, 0.5, 1, 1.5, 2, 2.5, and 3 mgL^{-1}) and supplemented with 3% sucrose and 7.5 gL^{-1} agar (A7921, Sigma-Aldrich).

Rooted plantlets that were two months old and about 15 cm tall were gently removed from test tubes, their roots were washed free of agar, and they were transferred to 13 cm diameter plastic pots (Hak plastik, Turkey) filled with autoclaved peat and perlite (1:1, v/v), at one plant per pot. Potted plants were covered with transparent plastic to prevent the loss of humidity and placed in a culture room (40 µmol m^{-2} s^{-1}, 25°C, 16-h photoperiod) and leaves were sprayed with water three times a day (400 mL $week^{-1}$ $plantlet^{-1}$). After 3 weeks, plantlets were transferred to 45-cell plug trays (1 cell = 80 ml) in a greenhouse (30°C; 2-11 MJ m^{-2} day^{-1}). After 10 days, the number of acclimatized plants and survival percentage were determined.

The experiment was set up as a completely randomized design (10 replicates × 6 combinations of plant growth regulators (PGRs)). Data was statistically analysed using the JMP® program ver. 5.00 (SAS Institute, Cary, NC). Means were separated according to the least significant difference (LSD) test at the 0.05 level of probability. Percentage values were all arcsine transformed prior to analyses.

3. Results and Discussion

Shoot including apical buds were cut into segments 2 cm long and placed in MS basal medium containing different combinations of BA and GA_3 to stimulate shoot growth and development (Figure 1A, B). The most effective combination of PGRs for micropropagation was 0.5 mgL^{-1} BA and 0.2 mgL^{-1} GA_3 (Figure 1C, D). After the first subculture, most shoots (6.03 shoots/node) formed in response to 0.5 mgL^{-1} BA and 0.2 mgL^{-1} GA_3, but after the second subculture, a maximum of 4.42 shoots/explant formed on the same medium (Table 2). Shoots formed in 85% of explants averaged over the first and second subcultures (Table 2). Following this pulse, and after the second subculture, plantlets were transferred to rooting media containing different concentrations of IBA (Figure 1E). IBA at 1.5 or 2.5 mgL^{-1} induced most roots (in 62.5% of shoots) (Figure 1F, G; Table 3). In the control (no auxin), no roots formed (Table 3). Plants were acclimatized in peat and perlite then adapted to soil (Figure 1H, I). Plantlets derived from rooting medium with 1.5 mgL^{-1} IBA showed best acclimatization (79.1% survival) (Table 4).

Figure 1. *Stages of Origanum sipyleum L. micropropagation. A) Donor plantlet. B) Four-week old micropropagated plants in Magenta vessels containing MS medium with BA and GA_3. C) Eight week old plantlets on MS medium with 0.5 mgL^{-1} BA and 0.2 mgL^{-1} GA_3. D) Shoots 5 cm long separated to induce multiple shoots. E) Multiple shoot formation in MS medium with 0.5 mgL^{-1} BA and 0.2 mgL^{-1} GA_3. F) Individual shoots induced to form roots on MS medium with IBA. G) Rooted plantlet 15 cm tall and four months old. H) Acclimatized plantlets 8 cm tall and about five months old. I) Plantlets adapted to soil in plug trays.*

Table 2. *Micropropagation of Origanum sipyleum L.*

Plant growth regulator and concentration	No. of shoots[a] after 1st subculture	No. of shoots[a] after 2nd subculture	Shoot regeneration (%)
0.1 mgL^{-1} BA + 0.1 mgL^{-1} GA_3	2.87 ± 0.39 c	2.01 ± 0.40 c	33.40 e (35.28)[b]
0.1 mgL^{-1} BA + 0.2 mgL^{-1} GA_3	3.90 ± 0.68 c	2.66 ± 0.66 b	40.40 d (39.46)
0.5 mgL^{-1} BA + 0.1 mgL^{-1} GA_3	4.00 ± 0.79 b	3.00 ± 0.44 ab	41.80 d (40.27)
0.5 mgL^{-1} BA + 0.2 mgL^{-1} GA_3	6.03 ± 1.23 a	4.42 ± 1.54 a	85.00 a (67.24)
1.0 mgL^{-1} BA + 0.1 mgL^{-1} GA_3	5.09 ± 1.61 a	4.22 ± 0.84 a	65.00 b (53.73)
1.0 mgL^{-1} BA + 0.2 mgL^{-1} GA_3	4.42 ± 1.35 ab	4.00 ± 1.14 ab	50.80 c (45.45)

Different letters within a column are significantly different (LSD test; $P \leq 0.05$)
[a]SE: Standard error.
[b]Values in parentheses indicate arcsine transformed % values.

Table 3. *Rooting of Origanum sipyleum L.*

IBA concentration	% Rooting of plantlets derived from nodes
0 mgL^{-1}	0
0.5 mgL^{-1}	27.5 c (31.39)[a]
1 mgL^{-1}	42.5 b (40.61)
1.5 mgL^{-1}	62.5 a (52.55)
2 mgL^{-1}	35.0 bc (36.22)
2.5 mgL^{-1}	62.5 a (52.55)
3 mgL^{-1}	47.5 b (43.55)

Different letters within a column are significantly different (LSD test; $P \leq 0.05$)
[a] Values in parentheses indicate arcsine transformed % values.

Table 4. *Number of acclimatized plants and their survival %.*

IBA concentration	No. acclimatized plants (A)	No. surviving plants (B)	Survival (%)	B/Total *100 (%)
0 mgL^{-1}	0	0	0	0
0.5 mgL^{-1}	8	2	25	1.1
1 mgL^{-1}	24	10	41.7	5.7
1.5 mgL^{-1}	48	38	79.1	27.2
2 mgL^{-1}	16	5	31.25	2.8
2.5 mgL^{-1}	42	23	54.7	13.1
3 mgL^{-1}	38	13	34.2	7.2
Total	176	91	51.7	

Our study indicates that the BA+GA_3 combination resulted in the formation of at least 6 shoots/node after the first subculture, without tissue browning, and that these shoots could be effectively rooted in 1.5 mgL^{-1} IBA. Rooted plantlets could then be effectively acclimatized. This protocol thus presents a simple and effective micropropagation protocol for endemic Turkish *O. sipyleum*. BA at 1 mgL^{-1} was essential to induce shoots from the apical shoot tips of seed-derived *O. sipyleum*, but only when 550 mgL^{-1} $CaCl_2$ was also added to MS medium [10].

The micropropagation of *O. minutiflorum* [12] and *O. acutidens* [4] required BA and NAA to induce shoots, then plantlets. In the former study, leaf segments and shoot explants (hypocotyls, single nodal segments and shoot tips) excised from seedlings were cultured on MS and B5 [13] media containing different combinations of BA and NAA: MS with 2.0 mgL^{-1} BA and 0.1 mg L^{-1} NAA induced most shoots (0.8/explant) while B5 medium was ineffective. In the latter study, nodes cultured on MS medium 1.8 mgL^{-1} BA and 0.2 mg L^{-1} NAA formed most shoots (8.5/explant). In contrast, Iconomou-Petrovich et al. (1998) found B5 medium with 0.1 mgL^{-1} NAA and no BA could effectively induce shoots from single node explants in *O. vulgare* subs. *hirtum* [14]. Leelavathi et al. (2013) found that MS medium with 2 mgL^{-1} BA and 0.5 mgL^{-1} 2, 4-D induced most shoots (not quantified) from *in vitro* apical buds of *O. vulgare* [15]. Both Morone-Fortunato et al. (2008) and Lattanzio et al. (2009) induced multiple shoots from axillary buds of *O. vulgare* subs. *hirtum* on MS and Nitsch and Nitsch media with 1 mgL^{-1} BA, extracting secondary metabolites in the latter, and inducing approx. 3 shoots/explant in the former study [16, 17]. Ueno and Shetty (1998) also found that 1 mgL^{-1} BA was best for the micropropagation of *O. vulgare* [18]. Goleniowski et al. (2003) induced as many as 22.2 plantlets/node in the presence of 0.06 mgL^{-1} BA and 0.1 mgL^{-1} NAA when single nodes of *O. vulgare* × *applii* were used [19]. Socorro et al. (1998) also described micropropagation from nodal segments with two axillary buds obtained from seedlings of *O. bastetanum* in the presence of 0.05 mgL^{-1} BA [20].

In the present study, best rooting was observed in MS medium with 1.5 or 2.5 mgL^{-1} IBA, inducing rooting in 62.5% of plants, in contrast to 0.5 mgL^{-1} IBA found by Oluk and Çakir (2009), where 96% of shoots rooted [10]. Rooting was spontaneous in *O. vulgare* [19] while *O. acutidens* rooted best in MS medium with 0.2 mgL^{-1} NAA with 1.8 mgL^{-1} BA [4]. *O. vulgare* rooted best on B5 medium with 0.01 mgL^{-1} NAA (Iconomou-Pethrovich et al. 1998) while 3.0 mgL^{-1} IBA in MS medium was needed for rooting of *O. minutiflorum* (Özkum 2007). Arafeh et al. (2003) rooted 90% of *O. syriacum* shoots on MS medium with 0.8 mgL^{-1} IAA [12, 14, 21].

4. Conclusion

Micropropagation is an important technique for preserving endemic Turkish *O. sipyleum* L. The use of BA with GA_3 to induce shoots from nodes, followed by the application of IBA to root shoots, results in a simple, but effective protocol to mass produce this aromatic herb.

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